Tissue Engineering in Achilles Tendon Reconstruction; the role of Stem Cells, Growth Factors and Scaffolds

Running title: Achilles tendon reconstruction

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Abstract

Achilles tendon injuries are common, and present a challenge in the acute and chronic setting. There is significant morbidity associated with the injury and the numerous management strategies, as well as financial implications to the patient and the health service. To date, repair tissue from all methods of management fail to achieve the same functional and biomechanical properties as the native tendon. The use of tissue engineering technology may reduce morbidity, improve the biomechanical properties of repair tissue and reduce the financial burden. The goal is to produce completely integrated tendon repair tissue that has the functional and mechanical properties of the native tendon. This review evaluates the role of stem cells in tissue engineering for tendon reconstruction and the various sources for harvesting stem cells. They can be obtained from the embryo, foetus or adult, and require the correct conditions for proliferation and differentiation. There remain many ethical concerns with the use of embryo or foetus harvested stem cells, thus the focus remains on adult sources, haematopoietic and non-haematopoietic. The improving knowledge of the role of growth factors is addressed, as is their effect on animal models for tendon repair. Growth factors include bone morphogenic proteins, transforming growth factor β, insulin-like growth factor and platelet derived growth factor. The role of scaffolds in human and animal models is reviewed, both naturally derived and synthetic scaffolds. Whilst numerous animal studies have reported encouraging results, further work is required.

Keywords

Achilles; tendon; stem cells; tissue engineering; pluripotent; differentiation; scaffolds.

Introduction

The Achilles tendon, formed by the union of the medial and lateral heads of the gastrocnemius muscle with the soleus muscle, is the strongest tendon in the body and inserts on the calcaneum [1]. The main arterial supply arises from the posterior tibial artery, which supplies the proximal and distal thirds of the tendon. The middle third, supplied by the peroneal artery, is relatively hypo-vascular and is a common site for tendon degeneration and rupture [2]. Achilles tendon rupture is reported to have an incidence of 11.3/100,000 per year [3].

Tendons are composed of a cellular component (fibroblast cells) and extracellular matrix. Water forms the majority of the extracellular matrix (70%); with collagen, ground substance and elastin composing the rest [4].

Within the human body, collagen types I, II and III are most frequently found, with type I dominating in tendons due to its high tensile strength. Type III collagen tends to be seen in greater proportion in healing tendon tissue [5]. A tendon attaches muscle to bone, transferring muscular force to the attached bone that can produce movement. Collagen, a triple-stranded helical molecule, is organised into parallel bundles of fibrils orientated to the direction of force. These fibrils group together to form fascicles that are enveloped by connective tissue layers. The vascularity of tendons is dependent on whether they are enclosed in sheath; with those within a sheath having avascular regions compared to those without a sheath. The vascular supply is from the musculo-tendinous junction, osteo-tendinous junction and vesicles within surrounding soft tissues. Within the endotenon, longitudinal arranged vessels are seen, which anastomose with periosteal vessels at the bone insertion site. In those tendons enclosed in a sheath, vessels enter along the tendon via vinculae in a segmental distribution, with adjacent areas supplied by diffusion. Those without a sheath have blood vessels passing into the tendon at any point through the paratenon [6].

Injuries to the Achilles tendon heal through three stages: haemostasis and inflammation, matrix and cellular proliferation, and maturation. During the first stage of repair the edges of the injury site are stabilised by formation of a fibrin clot following inflammatory cell and platelet aggregation. Fibroblasts then infiltrate and proliferate at the site of injury, producing extracellular matrix. Damaged tendons heal by scar formation that has inferior mechanical properties compared to the native tendon. Finally, matrix metalloproteinase degrade the collagen matrix, and collagen type 1 replaces collagen type III. These collagen fibres are organised in alignment of the direction of force [4].

The management of Achilles tendon injuries is dependent on the severity and location of the rupture. Non-operative measures may include splintage, exercises and pain management. Operative treatment options include debridement, repair and reconstruction with graft. The current treatment modalities have limited clinical evidence and have a significant complication and failure rate [3]. The goal is to be able to produce completely integrated tendon repair tissue that has the functional and mechanical properties of the native tendon. Given the lack of clinical evidence of current treatment options as well as associated morbidity and financial implications, research into the application of stem cells for cell-based strategies have gained momentum in recent years.

Stem cells

Stem cells can be obtained from the embryo, foetus or adult, and can reproduce for long periods under correct conditions. The stem cell develops by proliferation and differentiation, and each cell division being either symmetrical or asymmetrical. Symmetrical division means either two stem cells are produced or two terminally differentiated cells have resulted. With asymmetrical division the result is one stem cell and one terminally differentiated cell [4]. With increasing cell divisions the differentiation potential declines [7]. Embryonic stem cells, originating from the inner cell mass of the blastocyst, are harvested from eggs that are fertilized *in vitro*. They have pluripotent potential and can differentiate into any lineage. As the embryo develops the cells being produced demonstrate reduced phenotypic potential, limited degrees of differentiation and self-renewal potential, with greater rate of cell proliferation [7]. There are many ethical concerns associated with the use of stem cells from the embryo or foetus, and thus preclude their clinical use. Adult stem cells, haematopoietic or non-haematopoietic, maintain their multilineage potential [8].

Mesenchymal stem cells (MSCs) are multipotent, non-haematopoietic adult stem cells that are derived from the mesodermal germinal layer [9,10]. MSCs have multilineage potential with the ability to differentiate along chondrogenesis, adipogenesis and osteogenesis lineages [11]. MScs were first reported in bone marrow by Friedenstein *et al.* [12], and demonstrated their intrinsic ability to adhere to tissue culture plastic that enabled isolation. Further analysis found these cells to be a heterogeneous population of stem cells that were at different stages of differentiation potential [13,14]. The cell surface antigens on these non-haematopoietic stem cells allow them to be distinguished from haematopoietic stem cells [13,14]. They have higher levels of proliferation and collagen synthesis and survive longer compared to terminally differentiated cells. MSCs also have a low risk of immune reaction in the host whether autologous or allogenic, as they express no class II major histocompatibility complex (MHC).

A number of studies have reported encouraging outcomes in tissue regeneration using MSCs with scaffolds in animal models. Achilles tendon ruptures in rabbits treated with MSCs demonstrated significantly greater structural and material properties compared with controls [15]. With the injection of chondrocytes or MSCs there was improved healing at the Achilles tendon-bone interface in rat models, with the new enthesis organised like native enthesis tissue in the MSCs group [16]. Whilst Juncosa-Melvin *et al.* [17] reported greater maximum force and stress in MSCs seeded autogenous tissue-engineered constructs compared with constructs without

MSCs in repair of rabbit patella tendons. Awad *et al.* [18] also reported significantly improved repair in those that had MSCs injected into rabbit patella tendon defects. Ligament integration in bone tunnels and biomechanical strength were improved with MSCs coated or incorporated in grafts [19,20].

The mechanism of action of stem cells is still not fully understood. Whilst it is assumed that the stem cells undergo incorporation and proliferation in the host, alternate mechanisms of action exist. Some studies have shown that only a small proportion of the cells persist at the transplant site, and that the action of the MSCs may be due to the local release of paracrine factors with angiogenic, trophic and anti-inflammatory properties.

Sources of MSCs

Bone marrow

Bone marrow contains both MSCs and haematopoietic stem cells. Although bone marrow derived MSCs (BMSCs) form <0.01% of the nucleated cells obtained in bone marrow aspirates, they have been shown to have the greatest potential for differentiation into multiple cell lines. However, the technique of bone marrow aspiration itself is painful and may cause donor site morbidity [10].

Lee *et al.* [21] characterized the differentiation of BMSCs into tenocyte-like cells in the presence of bone morphogenetic protein-12 (BMP-12). They found that exposure to BMP-12 markedly increased the expression of scleraxis (Scx) and tenomodulin (Tnmd), which are markers of tenocyte lineage. Subsequent colonies demonstrated a persistent elevation in these markers. In addition, they found that BMSCs seeded in collagen scaffolds and similarly treated also expressed high levels of Scx and Tnmd, as well as type I collagen and tenascin-c. When these scaffolds were implanted into surgically created tendon defects in vivo, they generated tendon-like tissue with an increase in cell numbers, which were aligned along the tensile axis. Increased matrix deposition and elevated expression of tendon markers was also demonstrated.

Huang *et al.* [22] studied the effect of BMSCs in a rat Achilles tendon injury model. The cut Achilles tendons in the rats were injected with hypoxic BMSC, normoxic BMSC, or not treated. They demonstrated that BMSCs

improved the ultimate load to failure, with higher loads to failure in the group treated with hypoxic BMSCs. The BMSC groups had stronger immunostaining for type I and type III collagen, and bromodeoxyuridine (BrdU) labelling of the stem cells showed retention of the injected cells at the site of the tendon rupture.

The effects of MSCs on tendon healing in a rat tendon model has been reported [23], where the groups were randomized into suture repair only, suture repair with MSC injection, and repair with suture loaded with stem cells. There was significantly higher ultimate failure strength in both the MSC groups, with better maintenance of strength and histology in the suture loaded with stem cells group.

Adipose Tissue

Adipose tissue contains a population of stem cells that are similar to BMSCs. The advantages of adipose derived stem cells include the ease of harvesting, ready availability and low donor site morbidity. They have been shown to have multilineage potential by Zuk *et al.* [24]

James *et al.* [25] studied the effect of growth differentiation factor 5 (GDF-5) on rat adipose tissue-derived mesenchymal stem cells (AT-MSCs) that were cultured on a poly(DL-lactide-co-glycolide) (PLGA) fibre scaffold, which mimics the collagen structure of native tendon, as well as on a PLGA 2D film scaffold. They found increased expression of tendon markers including Scx, as well as genes for Type I collagen. In addition, the AT-MSCs cultured on the fibre scaffold showed increased expression of these markers when compared to those cultured on 2D film.

A study looking at the effect of AT-MSCs in a rabbit Achilles tendon healing model comparing platelet-rich plasma (PRP) gel and autologous AT-MSCs mixed with PRP was reported by Uysal *et al.* [26] At 4 weeks the AT-MSCs mixed with PRP group had a higher tensile strength compared to the PRP gel group. There was also an increase in collagen type I, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), and a decrease in transforming growth factor beta (TGF-β) in the AT-MSC treated group. There was increased

neovascularization in tendons treated with AT-MSCs on doppler ultrasonography as well as histologic analysis in an equine superficial digital flexor tendon (SDFT) model study by Conze *et al.* [27]

Synovium

The synovium is a readily accessible tissue for harvesting, with minimal donor site morbidity. Ju *et al.* [28] investigated the use of synovial MSCs in the healing of an Achilles tendon graft into a bone tunnel in a rat model. They found the labelled MSCs to be localized at the bone-tendon interface. There was accelerated tendon to bone integration in the MSC group, with an increase in the proportion of oblique collagen fibres (Sharpey's fibres) in the MSC group. Synovial derived MSCs have been reported to have superior chondrogenic potential than those from other sources [29]. These effects may have relevance in the treatment of insertional Achilles tendinopathy.

Peripheral Blood

MSCs are found in the circulating blood. Raghunath *et al.* [30] cultured peripheral blood mononuclear cells and found that there is a distinct cell group which could be cultured that were positive for cell surface markers CD105 and CD14 and expressed collagen I and II precursors. These cells did tend to demonstrate chondrogenic potential, however a variety of connective tissue could be produced in the correct environment. Collagen type II tissue has been produced from isolated adult pluripotent cells from peripheral blood monocytes [31]. Giovannini *et al.* [32] demonstrated that equine fibroblast–like cells derived from the peripheral blood can be directed towards adipogenic, chondrogenic and osteogenic differentiation.

It has been suggested that peripheral blood MSCs may actually be derived from the vascular tissue, and tends to yield lower levels of MSCs [33]. Umbilical cord derived MSCs has also been suggested as a good source, with shorter doubling times and longer time to senescence if compared to BMSCs. The role of peripheral blood derived MSCs in tendon repair and regeneration is yet to be determined.

Muscle/Periosteum/Tendon

Periosteum contains multipotent, mesodermal cells with the potential to form a variety of connective tissues. Tawonsawatruk *et al.* [34] studied the growth kinetics of rat mesenchymal stem cells derived from bone marrow, periosteum and adipose tissue and found that periosteum derived stem cells were comparable to BMSCs with similar growth curves and population doubling times. Kisiel *et al.* [35] compared the proliferative capacities of bone marrow-, adipose tissue-, muscle-, and periosteum-derived MSCs from canine muscle and periosteum and found that all had similar proliferative capacity, however periosteum provided a significantly higher MSC yield per gram of tissue. The use of periosteum at the graft-bone interface has been suggested to augment tendon repair. A study by Chen *et al.* [36] found that a periosteum cover over transplanted tendons in rabbit tibial tunnels formed a fibrous layer interface. This interface subsequently became a fibre-bone intermixture and anchorage, with progressive incorporation and organisation.

The role of muscle derived MSCs have been investigated in animal models by Wada *et al.* [37]. It was reported that they underwent terminal osteogenic differentiation *in vitro*, with bone formation. Tendon derived stem cells (TDSC) have also been evaluated, with Cheng *et al.* [38] suggesting that BMSCs had better osteogenic potential than anterior cruciate ligament (ACL) derived MSCs. Although, these ACL derived MSCs demonstrated faster proliferation and with basic fibroblast growth factor (bFGF) could maintain an undifferentiated state. Thus, better outcomes were reported from ACL derived MSCs compared to BMSCs. Zhang *et al.* [39] studied TDSCs and tenocytes isolated from the patellar tendon and Achilles tendon of rabbits, and found that TDSCs were able to differentiate into adipocytes, chondrocytes, and osteocytes *in vitro*, and form tendon-like tissues *in vivo*. In addition, TDSCs from patellar tendons formed more numerous and larger colonies and proliferated more rapidly than TDSCs from Achilles tendons. Tan *et al.* [40] compared rat TDSCs and BMSCs *in vitro*. They found that TDSCs exhibited higher clonogenicity, proliferated faster, and expressed higher TnmD, Scx, collagen 1 α 1 (Col1A1) than BMSC.

MSCs derived from bone marrow, synovium, periosteum, skeletal muscle and adipose tissue were compared by Sakaguchi *et al.* [29] It was found that cells derived from bone marrow, synovium and periosteum retained their expansion potential at later passages. There was greater colony numbers derived from synovium, periosteum, adipose tissue and muscle compared to bone marrow. BMSCs and periosteum derived MSCs were superior in osteogenesis, whilst synovium and adipose derived MSCs were superior in adipogenesis.

Growth Factors

Transforming Growth Factor β (TGF-β) family

These are a complex group of growth factors that are critical for embryonic development, which include the bone morphogenic proteins (BMP) and TGF-β which play a critical role in tendon and bone development.

The effect of Transforming Growth Factor β1 (TGF-β1) on the healing of rat Achilles tendon repair has been reported by Kashiwagi *et al.* [41] There was a dose dependent increase in the expression of procollagen type I and II messenger ribonucleic acids (mRNAs), and a higher failure to load and stiffness of the healing tendon in the TGF-β1 treated group. A study looking at the effect TGF-β1 on surgically transected rat Achilles tendons in rats using adenovirus-modified muscle grafts found accelerated healing of the tendon, with earlier return to normal histological appearance, accelerated restoration of mechanical strength, decreased deposition of type III collagen bundles and an increase in type I collagen bundles [42]. They also noted accelerated remodelling of tendon thickness.

A similar model was used to study the effect of BMP-12 on the healing of rat Achilles tendon, using a muscle flap modified by adenovirus carrying BMP-12 [42]. A higher load to failure was noted, as well as higher tendon stiffness and a more organized and homogeneous pattern of collagen fibres in the BMP-12 treated groups. There was also acceleration of tendon healing, with an earlier shift from fibroblasts to fibrocytes within the healing tendon. Pelled *et al.* [43] compared the effect of genetically modified MSCs over expressing BMP-2 and Smad8 with non–modified MSCs and controls in a mouse Achilles tendon repair model. They reported that the genetically modified MSC group showed a better material distribution and functional recovery than control groups, and also demonstrated the highest effective stiffness and elastic modulus.

The role of TGF-β and BMP-2 in injuries at the bone-tendon junction in a rabbit Achilles tendon model has been investigated by Kim *et al.[*44] who noted that the addition of TGF-β to fibrin glue did not improve the biomechanical properties of repair tissue. However, BMP-2 in combination with fibrin glue accelerated the

healing and also improved the histological and biomechanical properties of the repair. The effects of BMP-14 (Growth Differentiation Factor 5 (GDF5)) in a rat tendon suture repair model, with the suture acting as a carrier, were studied by Dines *et al.*[45]. Histologic analysis demonstrated improved healing in the GDF5 treated tendons by three weeks, as well as significantly higher ultimate tensile load and stiffness compared with controls, however there was no difference at six weeks.

Insulin-like Growth Factor (IGF) Family

IGF-I is a growth factor closely related to insulin that performs many functions, including mediation of growth hormone activity, stimulating proliferation of multiple cell types including tenocytes, and has been identified in tendons [46]. IGF-I has been shown to enhance collagen formation in the human patellar tendon [47].

Platelet Derived Growth Factor (PDGF)

PDGF is a glycoprotein that is required by various cell groups including fibroblasts for optimal growth and cell proliferation. PDGF-BB has been shown to induce collagen type I mRNA in equine tendon cultures [48]. Intra-tendon delivery of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) has been shown to cause a dose-dependent, transient increase in cell proliferation and sustained improvement in biomechanical properties of Achilles tendon repair in a rat collagenase-induced tendinopathy model [49]. rhPDGF-BB has also been shown to improve remodelling in a rat Achilles tendon transection model with a vicryl suture carrier, by significantly decreasing the resulting cross-sectional area, thus improving the material properties of the repaired tendon. In view of these findings, PDGF shows a promising role in treating tendinopathy.

Scaffolds

An appropriate scaffold in tissue engineering is key component to promote integration, growth and biomechanical support [50]. Collagen, silk and synthetic polymers are some of the biomaterials that have been investigated to date. An optimal scaffold should have the mechanical strength to allow immediate load bearing and integrate with host tissue, whilst balancing this with appropriate rate of biodegradation.

Synthetic biodegradable three-dimensional (3D) polymer scaffolds such as polylactic acid (PLA), polyglycolic acid (PGA) and PLGA have been investigated. These polymers degrade by hydrolysis and can be custom fabricated to match local environmental and mechanical properties. Lu *et al.* [51] produced a braided 3D scaffold of poly(L-lactic acid) (PLLA) to replicate the structure of native ligament collagen fibrils in a rabbit ACL model. The study reported a good amount of matrix formation when the scaffold was immersed in human recombinant fibronectin. Further work is being performed to determine the optimum braiding angle of the scaffold to accurately represent and produce native tissue [52]. Synthetic polymers may also take the form of gels or sponges. Sponge scaffolds have been fabricated from PLGA immersed in bovine collagen type 1 solution, creating a porous structure with the mechanical and cellular properties desired for tendon reconstruction [53]. In this study canine ACL fibroblasts were cultured on the scaffold *in vitro,* prior to rolling and implantation into mice, resulting in uniform matrix formation with cell viability.

The use of synthetic polymers allows the scaffolds to be fabricated into a structure similar in size and orientation as native collagen fibrils, with nanometre scale accuracy. The nanofiber scaffolds are reported to culture more collagen mass from seeded human ligament cells compared to that produced in randomly orientated scaffolds [54]. Composite scaffolds involving nanofiber (hydrophilic properties and high surface area) and microfiber (providing mechanical strength and degradation resistance) scaffolds have been investigated with seeded porcine BMSCs [55]. The results have been encouraging with high matrix production. Whilst current literature supports the use of aligned nanofiber scaffolds, further work is required to determine the ideal material, fibre diameter, braiding angle and spacing of fibres. One of the disadvantages of current synthetic scaffolds is that they may release by-products during degradation, which can enter the bloodstream and lead to toxicity. Scaffolds derived from natural tissue have this advantage over synthetic polymers and various studies have investigated their role.

The use of collagen type 1 as a scaffold for tendon and ligament reconstruction has been considered, however current technology is unable to organise the fibres into a biocompatible structure. Attempts to use this scaffold have resulted in a biomechanically weak structure [56]. Silk fibroin, demonstrates good tensile strength and toughness [57], has had encouraging results as a scaffold. This material has surface amino acids for cell adhesion (a property that synthetic scaffolds lack), it also degrades slowly and can be manufactured as a gel,

braided fibre or nanofiber [58,59]. Studies investigating braided silk fibres in scaffolds seeded with human BMSCs reported good cell numbers and matrix production with *in vitro* culture [60]. Composite naturally derived scaffolds are being investigated, with a combination of braided structures and sponge scaffolds of particular interest with good early results.

Decellularized tendon tissue is another naturally derived scaffold that maintains its native structure and composition, as well as the mechanical properties of the tendon extracellular matrix [61]. Farnebo *et al.*[62] reported that the use of decellularized Achilles tendon graft in a rat model resulted in improved mechanical properties and reduced immune response compared to untreated grafts. The role of acellular human tissue, in form of human dermal allograft, has also been studied in Achilles tendon healing. Cadaveric studies demonstrated improved mechanical strength [63] and no complications in small retrospective studies in patients [64,65].

Xenograft scaffolds have shown good initial results in tendon repair *in vitro.* Porcine small intestinal submucosa has been utilised as a scaffold for tissue engineering, demonstrating an ability to remodel into tendon tissue [66]. This is likely due to the retention of active growth factors that are involved in migration of cells to the scaffold [61]. This scaffold showed rapid degradation (60% by four months) and recruitment of marrow-derived cells that are involved in tissue remodelling [67].

Conclusion

Achilles tendon injuries, whether partial or complete, are common and carry significant morbidity and financial implications for the patient and health service. With current modalities the repair tissue rarely demonstrates the biomechanical and functional properties of native tendon. The role of tissue engineering in tendon repair or augmentation of repair has been investigated in animal models. The ideal source of MSCs still has not been agreed upon, and little is known regarding the signalling pathways involved in tenogenesis of MSCs. Whilst current studies have shown encouraging results with regards to improved biomechanical and histological properties, further work is required to ascertain the growth factors, biomaterials and source of stem cells required for tendon regeneration.

Conflict of interest

The authors have no conflict of interest to declare.

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